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Identification of Antigenic Sites on Three Hepatitis C Virus Proteins Using Phage-Displayed Peptide Libraries

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A novel approach to screening phage-displayed peptide libraries has been used to identify hepatitis C virus (HCV) core, NS4 and NS5 sequences, which are antigenic in humans. Two random peptide libraries were used for screening using a mixture of HCV-positive sera or individual antibodies to core, NS3, NS4, and NS5 HCV proteins affinity-purified from this mixture. Sequencing of 56 selected phage clones resulted in 28 different peptide sequences and identification of seven antigenic regions, three in the core protein (19-26, 34-49, and 73-83), three in the NS4 (1681-1693, 1712-1718, and 1726-1736) and one in the NS5 protein (2251-2260). No NS3-specific peptides were identified. The immune response to core, NS4 and NS5 proteins includes a variety of linear determinants whereas epitopes on the investigated part of NS3 protein appear to be conformation-dependant. ***J. Med. Virol. 56:105–111, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: antigenic determinant; epitope; vaccine; diagnosis; filamentous bacteriophage

INTRODUCTION

The hepatitis C virus (HCV), which causes a persistent infection and chronic liver disease, is an important risk factor in the development of hepatocellular carcinoma. Choo et al. [1989] cloned the infectious agent causing this disease. Intensive studies of HCV using recombinant protein technology led quickly to development of HCV diagnosis methods. Serological methods based on testing for specific antibodies in blood samples play the major role in modern HCV diagnosis and monitoring. In overall genome organisation and presumed method of replication, HCV is most similar to members of the Flaviviridae family. The HCV genome consists of ~9,400 bp positive polarity single-strand RNA, which encodes a single polyprotein of ~3,000 amino acids. This polyprotein after translation is cleaved into a series of structural and nonstructural proteins by a combination of viral and host cell proteases. The structural proteins are a capsid protein (core) and two envelope glycoproteins designated E1 and E2/NS1. The nonstructural proteins are NS2 through NS5 [Van Doorn, 1994]. Serodiagnosis of HCV is based on the use of recombinant proteins or synthetic peptides from different parts of HCV polyprotein. Different combinations of recombinant proteins and/or peptides corresponding to various regions of core, NS3, NS4, and NS5 proteins are generally used. Patients with hepatitis C usually produce antibodies against all viral proteins [Kleinman et al., 1992]. Knowledge of the antigenic structure of HCV proteins is important for correct choice of the optimal regions for use as diagnostic recombinant proteins. Currently, the antigenic determinants on all diagnostically used HCV proteins are well characterised. For this purpose, different methods of antigen mapping have been used. These include binding analysis of hepatitis C patients antisera to synthetic peptides [Okamoto et al., 1992; Simmonds et al., 1993; Goeser et al., 1994; Sallberg et al., 1994; Zhang et al., 1994; Khudyakov et al., 1995; Bhattacherjee et al., 1995; Pujol et al., 1996], or short HCV polypeptides expressed in prokaryotic systems [Goeser et al., 1994; Mondelli et al., 1994; Claeys et al., 1995].

At the present time, phage-displayed peptide libraries are used for antigen mapping [Barbas and Barbas, 1994]. The advantage of this approach is that it allows screening of huge libraries of peptide sequences by means of affinity selection of specific clones. The method is generally used for mapping of epitopes recognized by monoclonal antibodies [Pereboev and Morris, 1996], but it is possible to adapt this method for

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polyclonal antibodies [Folgori et al., 1994; Burton, 1995]. Prezzi et al. [1996] have used this approach to locate two antigenic sites on HCV proteins using a patient serum and a library of random peptides expressed in the pVIII protein of filamentous bacteriophage. In the present work, we have located HCV antigenic determinants using two different phage libraries of random peptides (pIII and pVIII), and we show that the number of epitopes identified can be increased by using affinity-purified antibodies.

MATERIALS AND METHODS

Lyophilised serum samples were obtained from rejected donor units and from patients shown to be hepatitis C antibody-positive by the "RecombiBest anti-HCV" kit ("Vector-Best," Koltsovo, Novosibirsk, Russia) and the 2nd generation HCV ELISA test (Abbott, North Chicago, IL). They were subjected to gamma irradiation (2 Mrad) to inactivate the virus. The cloning by RT-PCR of HCV cDNA fragments and the expression and purification of the corresponding are described in detail elsewhere (unpublished data). The recombinant proteins used is the present work correspond to the following HCV regions: Core, 1-124; NS3, 1201-1459; NS4, 1644-1812; NS5, 2052-2302. Amino acid numeration is given in accordance with Choo et al. [1991]. cDNA sequencing of these fragments revealed that we are dealing with HCV subtype 1b (data not shown).

Two phage-displayed random 15-mer peptide libraries were both a generous gift from Prof. George Smith (University of Missouri–Columbia). In the first library, peptides are displayed near the N-terminus of the pIII protein of the filamentous bacteriophage fUSE5. All five copies of the pIII protein contain the insert. In the second peptide library (constructed using the f88-4 vector), 15-mer peptide is exposed on the surface by the major phage coat protein pVIII. This phage has two copies of the pVIII gene, one intact and the other recombinant containing the insert. Such a construct needs no helper phage for replication and is able to express up to 20% of the recombinant protein [Smith, 1992].

Escherichia coli lysate for use as a blocking agent was prepared as follows: K91Kan E.coli cells from 20 ml of overnight culture were collected by centrifugation, resuspended in 1 ml TBS with 0.1% sodium azide, sonicated, and clarified by centrifugation.

The fUSE2 phage suspension blocking agent was prepared as follows: K91Kan *E. coli* cells were infected with the fUSE2 virions. The cells were grown overnight at 37°C in 250 ml LB medium with 20 μg/ml tetracycline and pelleted by centrifugation. The phage was PEG-precipitated from the supernatant, pelleted by centrifugation, and resuspended in 5 ml TBS with 0.1% sodium azide.

Affinity Purification of Antibodies

Antibodies specific to core, NS3, NS4, and NS5 were obtained by a micro-scale affinity purification method previously described [Du Plessis, 1995]. Each individual recombinant protein was electrically transferred from polyacrylamide gel onto a PVDF membrane (Immobilon-P, Sigma, St. Louis, MO). Each membrane was incubated separately with the mixture of anti-HCV sera and after washing, bound antibodies were eluted from the membrane at low pH.

Biopanning

The biopanning procedure was performed as described by Smith [1992], with modifications [Pereboev and Morris, 1996]. The first procedure, fmh;1.6qUSE5/15/SAB, used the 15-mer library on pIII and serum antibodies (SAB), whereas the second procedure, f88-4/15/AAB, used the 15-mer library on pVIII and the affinity purified antibodies (AAB) to individual HCV proteins. Anti-HCV antibodies (whole sera or affinity isolated antibodies) were first immobilised on the plastic surface of 35 mm sterile Petri dish by means of goat antibodies to human IgG (Vector Labs, Peterborough, UK) as described previously [Pereboev and Morris, 1996]. For fUSE5/15/SAB, the mixture of five HCV-positive sera was diluted 1:300 in TBST (Tris-buffered saline with 0.05% Tween-20). For f88-4/15/AAB, affinity-purified antibodies were diluted 1:20 in TBST. The plates were washed five times with sterile TBST, incubated with 10^8 TU of a phage library in 0.5 ml TBST for 1.5 h at room temperature with gentle rocking and then washed 10 times with sterile TBST. The bound phage was eluted with 0.4 ml 0.2 M Glycine-HCl, pH 2.2 for 10 min, and the acid was immediately neutralised with 1 M Tris-HCl, pH 9.0. The first round biopanning eluate was concentrated down to 0.1 ml with the use of Vivaspin-0.5 concentrators (Vivascience, Lincoln, UK) and used to infect "starved" *E. coli* cells [Smith, 1992]. For the infection, 50 μl of the concentrated eluate was mixed with 0.1 ml of bacterial suspension. The mixture was rocked for 10 min at room temperature, 1 ml of LB media containing 0.2 μg/ml tetracycline was added, and the culture was incubated for 30 min at 37°C. Half of the cell suspension was plated onto an LB-agar plate containing 20 μg/ml tetracycline to obtain single colonies. The second half was transferred into 20 ml LB medium supplemented with 20 μg/ml of tetracycline for amplification. When the f88-4/15/AAB system was used, up to 1 mM IPTG was added to the culture for induction of recombinant pVIII expression. The plates and the culture (with rocking) were incubated overnight at 37°C. The amplified culture was then centrifuged for 20 min at 2,000g and concentrated down to 1 ml using the Vivaspin-15 by centrifugation for 45 min at 2,000g. To the concentrate, 0.15 ml of PEG-8000 (20% in 2.5 M NaCl) was added, and this was left at 4°C overnight. The precipitate was pelleted by centrifugation for 10 min at 10,000g, resuspended in 0.1 ml TBS containing 0.1% sodium azide, and used for subsequent rounds of biopanning. Colonies on the agar plates were tested for HCV-specific phage by lift-immunoblotting [Wang and Meng, 1996] plates with 100–500 colonies using nitrocellulose mem-

branes (Schleicher and Schull, Dassel, Germany) and incubating the lifts with antisera. For subsequent detection of anti-HCV antibodies bound to the nitrocellulose, the "Biotinylated Goat-anti-Human IgG Antibodies—Avidin—Biotinylated Horseradish Peroxidase" system (VectaStain, Vector Laboratories, Burlingame, CA) was used with diaminobenzidine as substrate. To decrease the level of nonspecific binding, all the incubations were performed in blocking buffer: 0.5% casein in TBST with addition of an *E. coli* lysate (1:200) and a fUSE2 phage suspension (1:200)

The positive clones were then cross-tested in immunoblotting with the individual HCV-positive sera, with a set of HCV-negative sera and with purified antibodies to the individual HCV proteins. The cells tested were "streaked" onto a nitrocellulose membrane placed on top of the agar plates (up to 1 mM IPTG was added when the f88-4/15/AAB system was used). The membranes were analysed as described above. The positive clones were propagated in 2 ml LB medium containing 20 μg/ml tetracycline and phage were isolated by PEG-precipitation. Phage DNA was purified by phenol-chloroform extraction followed by ethanol precipitation [Smith, 1992]. The nucleotide sequence of specific inserts was established by dideoxy-sequencing using the primers 5′-CCCTCATAGTTAGCGTAACG-3′ for fUSE5/15/SAB and 5′-AGTAGCAGAAGCCTGAAGA-3′ for f88-4/15/AAB and Sequenase 2.0 (Amersham International, Little Chalfont, UK).

RESULTS

Affinity Selection in the fUSE5/15/SAB System

Five HCV-positive human antisera were selected for their ability to recognize all four different recombinant HCV proteins (core, NS3, NS4, and NS5) on Western blots (results not shown). Antibodies from a mixture of the five sera were captured onto a Petri dish, which had been coated with goat anti-(human Ig) immunoglobulins. Biopanning of the fUSE5/15-mer library was then performed with these captured antibodies. Figure 1 shows the increase in the number of positive clones between the second and third rounds of biopanning. Positive clones were re-screened using individual HCV-positive sera and a set of HCV-negative sera. Clones that reacted with at least some of the 5 HCV-positive sera and not with HCV-negative sera (Fig. 2) were examined further. Twenty-six clones were selected and their phage DNA was isolated, purified, and sequenced. Seven different peptide sequences were obtained (Table I). The clones were additionally cross-screened with affinity purified antibodies to individual HCV proteins core, NS3, NS4 and NS5 (Fig. 2). All the clones reacted with anticore antibodies, except one clone specific to NS4.

Affinity Selection in f88-4/15/AAB System

Because >90% of clones selected with whole antisera recognized anticore antibodies, affinity purified antibodies were used individually for biopanning to increase the range of antigenic peptides identified. Affinity purification of the mixture of HCV-positive antisera was performed on a micro-scale using recombinant HCV protein fragments attached to a PVDF membrane. Two rounds of affinity selection were performed in this f88-4/15/AAB system, separately with purified antibodies to core, NS3, NS4, and NS5. Selected clones were tested first for interaction with the mixture of HCV-positive sera as described for the fUSE5/15/SAB system. No clones selected with anti-NS3 antibodies were positive with anti-HCV sera. Several clones se-

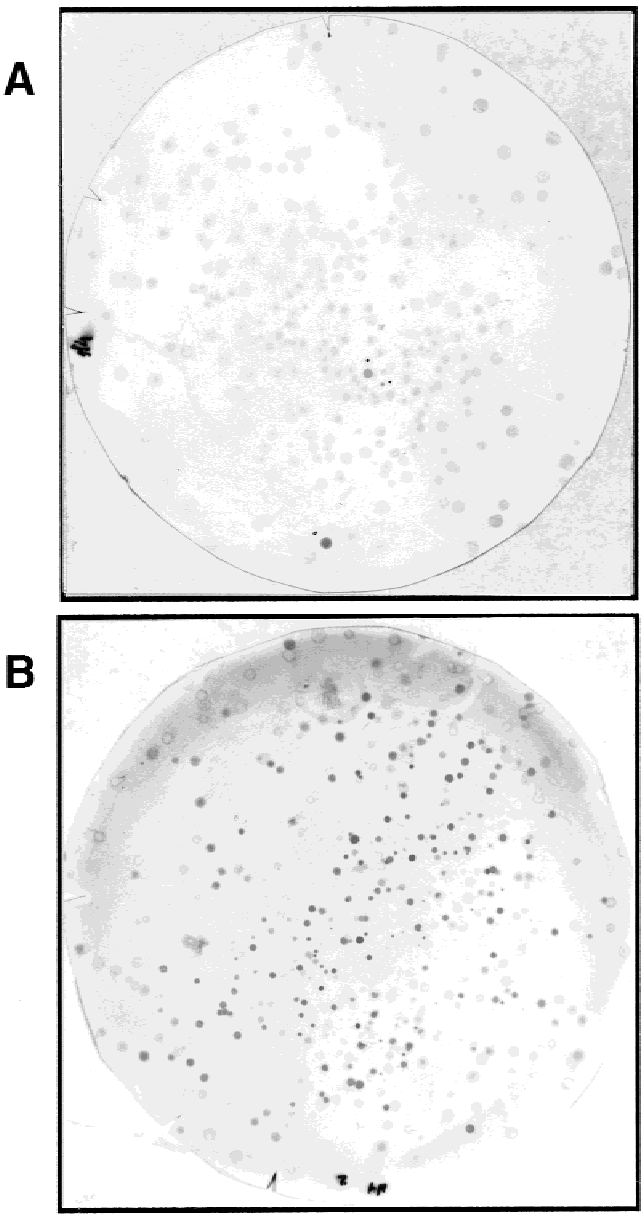

Fig. 1. Lift-immunoblotting of K91K *E.* coli cells infected with phage recovered from the 2nd **(A)** and 3rd **(B)** round of biopanning. A marked nitrocellulose membrane was placed on a culture of single colonies on an agar plate and lifted. The attached cells were washed away with TBST. The membrane was then blocked with TBST/casein and treated with the mixture of five HCV-positive sera. Specific binding was revealed using the Vectastain second antibody system and diaminobenzidine as substrate.

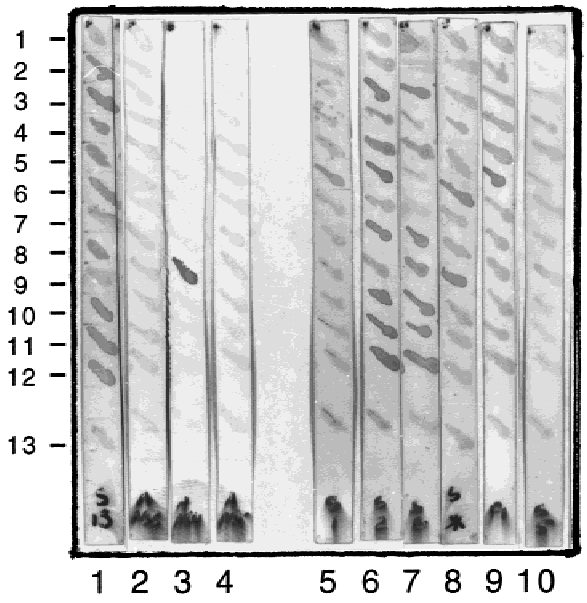

Fig. 2. Cross-screening of the clones selected in the fUSE5/15/SAB system. Twelve colonies that were positive after screening with the mixture of HCV-positive sera were streaked out on 10 nitrocellulose strips placed on tetracycline-containing agar and grown overnight. Cells were then removed from the strips, the membranes were blocked with TBST/casein and treated individually with affinity purified anticore, anti-NS3, anti-NS4, anti-NS5 antibodies (**lanes 1–4** respectively) and individual HCV-positive **(lanes 5–9)** and HCV-negative **(lane10)** sera. Vertical numbers 1–12 correspond to clones 2, 209, 3, 1, 5, 11c, 242, 70, 71, 46, 63, 178, respectively, in Table I, except that clone 242 does not appear in Table I because it was not sequenced. The bottom streak (13) is a negative control—K91Kan *E. coli* cells infected with fUSE2 virions (phage with no insert).

TABLE I. HCV-specific peptides selected in the fUSE5/15/SAB system*

Clones	Sequence
1, 2, 19 (3)	PPVLYLPWLPWGPSV
3, 26 (2)	HGRYFHVPGYPWGSA
5, 46, 169, 217, 218, 234, 282 (7)	PSDRLFPGLSDFGCS
63, 178, 11c, 12c, 21c–26c (10)	VYFLPLRGERVKSVD
70 (1)	LSDRLFPGLSHLDCS
71, 75 (2)	RGGDQPYIFLFAHIP
209 (1)	DLSVLGSRRPFFVWA

*Number of identical clones is shown in parentheses.

lected with antibodies to core, NS4, and NS5 proteins appeared positive with the anti-HCV sera. These were tested further using individual HCV-positive sera and individual antibodies to core, NS3, NS4, and NS5 (Fig. 3). Recognition of core-selected clones varied between different HCV-positive sera, indicating a diverse immune response to core protein (Fig. 3A, lanes 5–8). In contrast, all the NS4- and NS5-selected clones reacted with all four antisera tested, strongly in the case of NS4 and weakly for NS5 (Fig. 3B,C, lanes 5–8). Nevertheless, all NS4- and NS5-selected clones were strongly positive in reactions with corresponding affinity-purified antibodies (Fig. 3B,C, lanes 1–4). Thirty clones were chosen for sequencing: 7 clones strongly reacting only with anticore antibodies (Fig.3A), 11 clones reacting only with anti-NS4 antibodies (Fig. 3B), and 12 clones reacting only with anti-NS5 antibodies (Fig. 3C). The sequencing of these clones revealed 20 different sequences, 4 for core, 9 for NS4, and 7 for NS5 (Table II).

Sequence Analysis

Figure 4A shows the alignment of phage-displayed peptides with the core protein sequence. Three different antigenic determinants have been identified on this protein, involving amino acids 19-26, 34-49, and 73-83. Peptides selected in both fUSE5/15/SAB and f88-4/15/AAB systems took part in revealing these regions. We could not find a homology region for peptide 209 (Table I), although it reacted with anticore antibodies (Fig. 1). This peptide may be mimicking a conformational epitope formed by distant amino acids brought together by folding of the polypeptide chain. Peptides matching with NS4 and NS5 proteins are shown in Figure 4B,C. The sequence 1681-1736 contains three different antibody binding sites on the NS4 protein: 1681-1693, 1712-1718, and 1726-1736 (Fig. 4B). Six peptides were obtained from the f88-4/15/AAB system and one from the fUSE5/15/SAB system. It was not possible to establish any significant similarity for three of the peptides reacting with anti-NS4 antibodies (c. 3, c. 5 and c.30, 33 in Table II). Figure 4C shows a new antigenic determinant identified within amino acids 2251-2260 of the NS5 protein. However, five peptide sequences selected with anti-NS5 antibodies (c. 7, c. 9, c. 10, 13, c. 11 and c. 12, 14 in Table II) could not be matched with the HCV sequence, although there is a good similarity among themselves (Fig. 5).

DISCUSSION

There are both advantages and disadvantages to the use of phage display methods with sera from patients. First, serum antibodies are extremely heterogeneous and recognize a wide epitope repertoire. Because of this, special methods for peptide selection and for interpretation of results must be used. However, when patients' sera are used, an investigator is dealing with antibodies produced in humans in response to a real infection. Therefore, a unique opportunity exists to study the immune response to infection at the molecular level, without needing the etiological agent of the disease [Folgori et al., 1994]. The phage particles bearing specific peptides could be used directly for diagnosis. These advantages of using polyclonal antisera for screening phage-displayed peptide libraries are potentially valuable for diagnosis of infectious diseases and for vaccine design.

Prezzi et al. [1996] have also mapped HCV antigenic determinants using phage- displayed peptide libraries and they located two anti-HCV antibodies binding sites, consisting of amino acids 10-16 (core) and 1931-1938 (NS4). They screened the library with a single HCV-positive serum, whereas we have extended the potential epitope repertoire by using a mixture of five

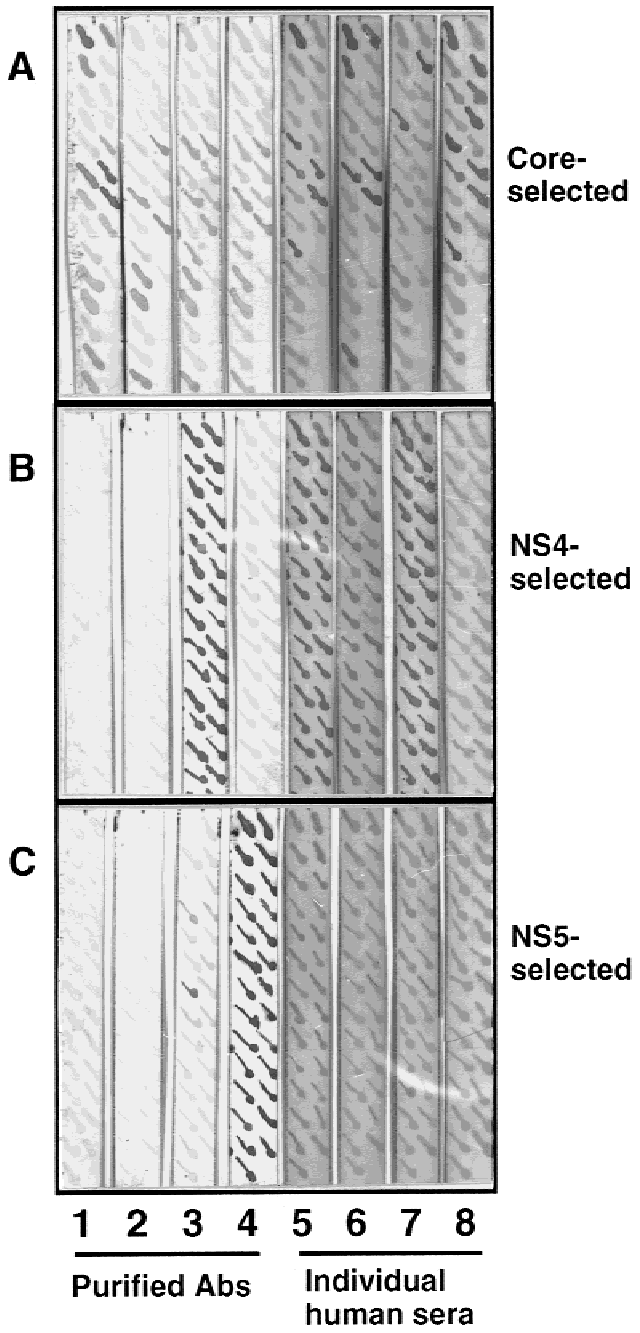

Fig. 3. Cross-screening of the clones selected in the f88-4/15/AAB system. Phage were selected using affinity purified antibodies specific to the individual HCV proteins (core, NS3, NS4, NS5) and first screened against the mixture of HCV-positive sera. Positive clones were then tested individually with affinity purified anti-core, anti-NS3, anti-NS4, anti-NS5 antibodies (**lanes 1–4** respectively) and individual HCV-positive sera **(lanes 5–8). A:** Clones selected with anti-core antibodies. **B:** Clones selected with anti-NS4 antibodies. **C:** Clones selected with anti-NS5 antibodies. No HCV-positive clones were selected using anti-NS3 antibodies.

high titre sera, each of which was known to react with four HCV proteins, and also by affinity purification of specific antibody components for biopanning. For affinity-purified antibodies we also used a library exposed on the pVIII surface protein rather than pIII because the former has many copies per virion compared with only five copies of pIII. Three rounds of biopanning were needed when unpurified sera were used (Fig. 1), and this may also have restricted the range of mimotopes identified by selecting for the strongest antibody-antigen interactions at the expense of weaker ones. The need for only two rounds of biopanning with purified antibodies may have contributed to the greater range of mimotopes identified with this system (Table II). There are some restrictions on the type of epitope likely to be identified by the peptide approach. Epitopes that cannot be mimicked by peptides, such as conformational epitopes or posttranslationally modified epitopes, are largely excluded. With affinity purified antibodies, there is the further restriction that the recombinant HCV protein fragments used for purification are only partial sequences and epitopes outside them will not be detected. Both sequences of Prezzi et al. [1996] differ from the seven we have identified, although their second sequence is outside the scope of our affinity-purified antibody selection method, since our NS4 protein fragment encompasses only 1644-1812 amino acid residues.

The HCV core protein has been well characterised antigenically using synthetic peptides, especially its N-terminal part [Okamoto et al., 1992; Goeser et al., 1994; Sallberg et al., 1994; Prezzi et al., 1996; Pujol et al., 1996]. The fact that it is possible to map core antigenic determinants using short peptides suggests a rather linear character for core epitopes. The antigenic regions 34-49 and 19-26 revealed in our study also have been identified using overlapping synthetic peptides. Antigenic core peptides include 20-41 and 40-63 [Goeser et al., 1994], 38-74 [Okamoto et al., 1992], and 45-64 [Pujol et al., 1996]. The specific binding site at 73-83 (Fig. 4A) has not been described previously. The variety of antigenic sites on the core protein identified with different sera suggests that much of its N-terminal part may be immunogenic, the immune response varying between individuals.

All our attempts using 15-mer peptide libraries to find peptide sequences mimicking NS3 antigenic determinants have failed, although only part of NS3 was investigated (amino-acids 1201-1459). This could be connected with the conformational character of NS3 antigenic sites, as suggested indirectly by studies in which anti-HCV antibodies bound only to longer fragments of NS3: 90 amino-acids [Mondelli et al., 1994], 85 amino-acids [Claeys et al., 1995]. Khudyakov et al. [1995] described 20-mer NS3 synthetic peptides interacting with anti-HCV antisera. However, that interaction was only observed in a very limited fraction of the sera (1 of 20, 2 of 20), which also might testify to a rather conformation-dependent character of NS3 antigenic determinant. The antigenic region 1681-1736 that we have identified in NS4 has also been located using synthetic peptides. Four different groups all located NS4 epitopes to within amino acids 1689-1730

TABLE II. HCV-specific Peptides Selected in the f88-4/15/AAB System

Clones	Sequence	Selected with
F88-4 CORE C. 1, 25 (2)	SRSPTRKKINNIILN	Anti-Core AB
F88-4 CORE C. 2, 16, 17 (3)	MPIRRKRKNIRTIIS	Anti-Core AB
F88-4 CORE C. 6	MPMRRKRKNIRSIVS	Anti-Core AB
F88-4 CORE C. 13	QSIRSTTRLHITLSL	Anti-Core AB
F88-4 NS4 C. 3	WSQMVPFDPLRLLDGS*	Anti-NS4 AB
F88-4 NS4 C. 5	SPVTLTESVFPFLR**	Anti-NS4 AB
F88-4 NS4 C. 6	TPGNHWGETTFPALR	Anti-NS4 AB
F88-4 NS4 C. 29	RHKIINIRPIRRIKL	Anti-NS4 AB
F88-4 NS4 C. 30, 33 (2)	HSIRSTTRLHTTLSL	Anti-NS4 AB
F88-4 NS4 C. 31	XRLTRRLSIHRIRLI	Anti-NS4 AB
F88-4 NS4 C. 32	SRKQKNIQLRSSNNS	Anti-NS4 AB
F88-4 NS4 C. 35, 21 (2)	SRKQKKIRLRSSNNT	Anti-NS4 AB
F88-4 NS4 C. 36	TPSTEQFDSFYDPLR	Anti-NS4 AB
F88-4 NS5 C. 1	XXXXXSPYILDFSPL	Anti-NS5 AB
F88-4 NS5 C. 3, 5, 6 (3)	ADDTPYTLSFAPHRH	Anti-NS5 AB
F88-4 NS5 C. 4	TPALNSLPYVFLFSY	Anti-NS5 AB
F88-4 NS5 C. 7	RHNTIRQQRRKNHHL	Anti-NS5 AB
F88-4 NS5 C. 9	NEVRSPRRGIKRHRV	Anti-NS5 AB
F88-4 NS5 C. 10, 13 (2)	TPIQTQRIKRHHLRR	Anti-NS5 AB
F88-4 NS5 C. 11	TTQRIINRRRRRRSR	Anti-NS5 AB
F88-4 NS5 C 12, 14	TQSRKTQSPMLIIQL	Anti-NS5 AB

*-Clone contains a 16-mer insert; **-Clone contains a 14-mer insert; X-The sequence was not read; AB-Antibodies.

Fig. 4. Alignment of phage displayed peptides with **(A)** HCV core, **(B)** NS4, and **(C)** NS5 proteins. The numbers are HCV amino acid positions according to Choo et al. [1991]. The following array of similarity of amino acids was used: A = G; K = H = R; D = E; S = T; N = Q; F = W = Y; P; C; V = I = M = L [Ivanisenko and Eroshkin, 1997]. * : Clone selected in fUSE5/15/SAB system on a mixture of anti-HCV-sera; ** : Clone selected in f88-4/15/AAB system using individual antibodies to core (A), NS4 (B), and NS5 (C) proteins; X :the sequence could not be read.

[Simmonds et al., 1993; Sallberg et al., 1994; Bhattacherjee et al., 1995; Khudyakov et al., 1995]. Our approach has identified one important residue just outside this region (Leu-1735; Fig. 4B) and several antigenic amino acids within it. The NS5 antigenic region 2251-2260 identified in this study has not been previously reported in literature, although several other NS5 epitopes have been identified [Zhang et al., 1994; Khudyakov et al., 1995; Pujol et al., 1996].

Although there is great similarity among the five

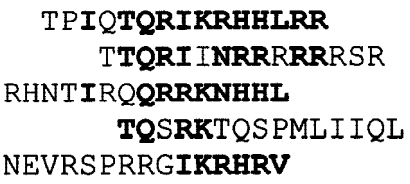

Fig. 5. Alignment of five peptide sequences, selected with anti-NS5 antibodies, which could not be matched with the HCV sequence.

peptide sequences in Figure 5 that react with affinity-purified anti-NS5 antibodies, they could not be matched at all with the NS5 sequence. This raises the possibility that we may have identified a conformational antigenic site in which the important amino-acid residues identified in Figure 5 are brought together by protein folding, rather than existing in HCV as a linear sequence. The ability to identify such antigenic sites by phage display methods would be important for diagnosis or prognosis of infectious diseases.

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